# Investigation of Microbial Ecology, Structure, and Function in Coalbed Aquifers: Powder River Basin, Montana

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# Abstract

In southeastern Montana, coal beds supply coal for energy; water for domestic and agricultural uses, and are being developed for coalbed methane (CBM). A comprehensive understanding of the relationships between the hydrogeologic systems and the total microbial community at depth will help establish best management practices for methane production.

The origin of CBM in the Powder River Basin (PRB) is the result of microbial processes (biogenic methanogenesis). The purpose of this research is to begin the process of identifying the structure, diversity and presumptive function of the total microbial community and ecology within a specific methane-bearing coalbed aquifer in the PRB and conduct culture-based investigations that will help delineate the kinetic rates and pathways for methanogenesis.

Samples of coal and coal-aquifer water were collected and analyzed. The coalbed water-quality sample is typical of CBM production water in the PRB, with a total dissolved solids concentration of 2,056 mg/L, specific conductivity of 3,275 umhos/$cm^2$, a sulfate concentration of less than 2.5 mg/L, and a sodium adsorption ratio of 25.5.

Results of total microbial community analyses from the aqueous phase indicate a relatively low diversity of the total community. Sequencing of several prevalent bands indicated that all presumptive identities, based on known sequences in the Ribosomal Database (RDP II), had relevant metabolic capabilities consistent for their presumed role in coal formations that generate methane.

The methanogen sequences, also derived from the aqueous phase, were closely associated with the genus *Methanolobus* within the order *Methanosarcinales*. In addition, all five organisms were most closely related to the species *M. taylorii* or *M. oregonensis* (averaging 93% homology). Interestingly and unexpectedly, this group is typically linked with marine environments, which may indicate that they thrive in a high sodium environment. However, the geological formation and shallow depth where this sample was taken have not been associated with ancient marine origins.

In addition, seven sequences were produced from a coal sample, and all are related to members of the *Methanosarcinales* or *Methanobacteriales* orders. Six appear to be unique from each other and their closest matches are to environmental clones from various methane-related origins.

In addition to the molecular data, coal samples were incubated in an anaerobic growth media used to specifically culture methanogens. To date this has proven to be unproductive. However, growth is typically very slow and may take a period of time well beyond the time frame of this study.

This initial investigation proved to be an excellent starting point for continuing efforts toward unraveling the complexity of the microbial community responsible for biogenic methane production.

# Introduction and background

Coal beds supply three critical resources in southeastern Montana: 1) coal for energy; 2) water for domestic and agricultural uses; and 3) coalbed methane. Currently, coalbed aquifers are being impacted by conventional coalbed methane (CBM) development. As concerns of global warming increase, speculation that these aquifers may serve as repositories for industrial $CO_2$ suggests that additional impacts are likely in the future. A comprehensive understanding of the relationships between the hydrogeologic systems and the total microbial community at depth will help establish best management practices for methane production and potential $CO_2$ sequestration. Minimizing waste of the methane and the production water is key to preserving the aquifers yet allowing for long-term methane production, and may help remediate the atmospheric $CO_2$.

Methane is held on cleat faces and micropore surfaces in coal by a combination of physical sorption and hydrostatic pressure from ground water in the coal (Law and others, 1991; Rightmire, 1984), and is released when the water pressure is reduced. To reduce hydrostatic pressure and capture released gas, water is pumped from wells drilled and completed in coalbeds.

The origin of CBM in the Powder River Basin (PRB) is the result of biogenic methanogenesis, a microbial process (Law and others, 1991). The success of $CO_2$ sequestration strategies may be a function of microbial activities as well. The purpose of this research is to begin the process of identifying the structure, diversity and presumptive function of the total microbial community and ecology within a specific methane-bearing coalbed aquifer in the PRB and conduct culture-based investigations that will help delineate the kinetic rates and pathways for methanogenesis. We foresee the value of data collected during this project as a means of moving toward a philosophy of harvesting CBM over long periods of time.

There are two distinct types of ground-water flow systems in the Powder River Basin, a deep regional system and a series of local flow systems. Ground water flows generally from the south to the north, with flow in the local systems reflecting topographic control. Ground-water recharge occurs at outcrop areas around the edges of the Basin in Wyoming and in high clinker-capped ridges such as the Wolf Mountains in Montana (Wheaton and Donato, 2004). Coal seams are the most continuous water-bearing geologic units and have hydraulic conductivity values equal to or slightly greater than those in sandstone aquifers.

Due to the geologic structure of the Powder River Basin, and the topographic relationship between generally higher elevations in Wyoming and lower elevations in Montana, coal seams crop out along valley walls in Montana and ground-water discharge areas are reflected in the springs that occur in these outcrop areas. Additional ground-water discharge occurs as baseflow to streams and rivers in Montana.

The quality of ground water in the Powder River Basin reflects chemical and biological reactions that occur along flow paths. In deep coal beds, such as those that contain coalbed methane, chemical reactions have greatly reduced the amounts of sulfate, calcium, and magnesium, and the water quality is dominated by moderate concentrations of sodium and bicarbonate. Coalbed methane can only exist in the sulfate-depleted, anoxic conditions which occur in deeper coals. All CBM production water is rich in sodium and much of it has a high SAR value and moderate concentrations of total dissolved solids (Van Voast, 2003).

It is understood that biogenic methane is produced as an end-product of a complex set of metabolic pathways represented by a consortium of microorganisms, including members of the domains *Eubacteria* and *Archaea*. This intricate and closely associated assemblage resides in anoxic zones depleted of typical electron acceptors (sulfate, nitrate, ferrous iron) found in many subsurface environments. Four groups of functionally diverse prokaryotes have been identified, as being necessary for the formation of methane under these conditions: 1) hydrolytic bacteria, 2) fermentative bacteria, 3) acetogenic bacteria and 4) methanogens (Whiticar, 1999). Each of these groups of microorganisms is responsible for an important function in the methanogenic pathway.

The hydrolysis of higher molecular weight substrates, such as cellulose, high molecular weight proteins and mixed composition polysaccharides by hydrolytic and cellulolytic competent bacteria is a necessary first step in the decomposition of organic materials (Figure 1). It has been postulated that this process is the rate limiting step in the formation of methane in anoxic environments. Following their breakdown into monomeric subunits such as short-chain fatty acids, sugars, amino acids and additional substrates (e.g. ammonia and hydrogen sulfide), fermentation proceeds, mediated by assorted fermentative bacteria.

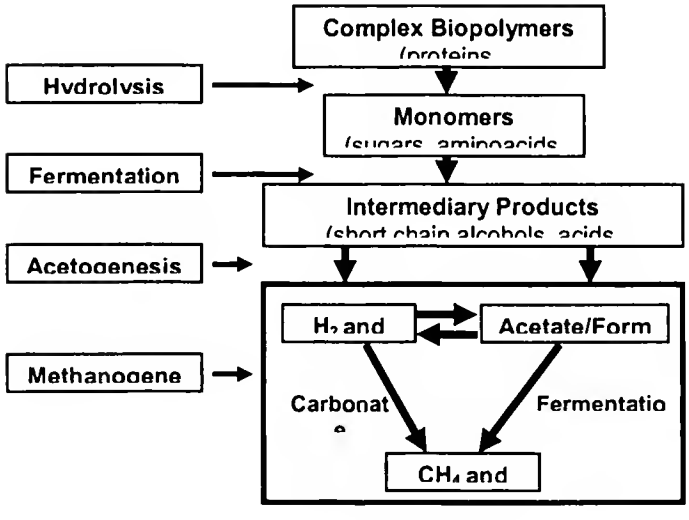


Figure 1. Anaerobic degradation of organic

The fermentation process produces a number of byproducts, including additional short-chain alcohols and acids (propionate and butyrate are common), as well as, acetate, formate and carbon dioxide ($CO_2$). Because methanogens metabolize a narrow range of compounds and are restricted to anoxic environments with redox potentials of Eh < -200 mV (Budwill, 2003), some further degradation is assumed to be required. Syntrophic acetogenic

bacteria play an important role in consuming many of the short-chain acids that accumulate in the pathway, and the end products, predominately acetate and $CO_2$, become viable substrates for methanogenesis.

The final step prior to conversion to methane is to convert any remaining alcohols and acids into acetate, carbon dioxide (methanol and methylamines may also be substrates for methanogenesis), hydrogen ($H_2$) and in some cases formate. This general scheme of anaerobic degradation of organic compounds to methane is diagrammed in figure 1.

Although the reduction of carbon dioxide by hydrogen (equation 1) is thought to be the most commonly used method for the production of methane in anoxic environments (Scott, 1999), the reduction of acetate, or a very limited methyl group containing hydrocarbon (equation 2) provides a greater change in free energy and therefore is more favorable for energy conservation. This situation remains unclear, as the opposite is thought to be true in certain environments such as marine or open freshwater settings (Whiticar and others, 1986). The two pathways may operate simultaneously under some circumstances and at differing stages of sedimentation of organic materials (Kotelnikova, 2002). While each of the two reductive processes produces methane, the two separate pathways may be active. The former by the (hydrogen mediated reduction of carbon dioxide) carbonate reduction pathway and the latter by the fermentation pathway. The general chemical equations for the respective pathways are illustrated below.

Equation 1:

Carbonate reduction pathway: $4\ H_2 + HCO_3^- + H^+ = CH_4 + 3H_2O$ ($\mu G°$ -3.2 KJ)*

Equation 2:

Fermentation (methyl-group) pathway: $CH_3COO^- + H_2O = CH_4 + HCO_3^-$ ($\mu G°$ -24.7 KJ)*

*Reported free energy values vary from source to source.

Evidence supporting the notion that the two pathways operate at various times was reported by Chin and others, (2003). They described temporal changes in methanogen production in flooded rice paddies. Their findings indicate that structural changes in the methanogenic community lead to functional changes in methane production with time. Similarly Scheid and others (2003) using rice roots as a community model for methanogenesis were able to show methanogenic community shifts when nitrate and sulfate were introduced. Apparently, the addition of alternative electron acceptors leads to changes in community substrate usage and may have broad effects on

community structure and activity. Likely, competition between methanogens and sulfate- and nitrate-reducing bacteria led to these changes.

The use of culture-independent molecular techniques for our investigation is crucial. It is generally accepted that classic culturing techniques may under represent microbial diversity in typical environments by two to three orders of magnitude (Torsvik and others, 1990a; Torsvik and others, 1990b). It is apparent that microbial communities and their associated populations play important roles in biogeochemical and physicochemical processes including methanogenesis and carbon cycling. Functional guilds of bacteria that have been associated with biogenic methane production include hydrolytic and cellulolytic bacteria, fermentative and acetogenic bacteria, as well as methanogens (Whiticar, 1999). However; Polman and others (1993) reported that there were no viable microorganisms in three different ranked coals. Their observations were based on results of experiments attempting to grow bacteria in cultures. Vorres (1990) reported that anaerobically preserved coals produced methane in sealed ampoules. Also these samples contained cultivable Clostridium species. In 1994, work by both Johnson and others (1994), and Volkwein and others (1994), noted that higher-rank coals produced low-molecular-weight organic acids when they were inoculated with presumptive anaerobic consortiums from various sources. Based on additional work completed by these groups, they concluded that the microorganisms collected from those environments (that were likely to contain methanogens and other consortium members) were responsible for the production of the methane. In Volkwein and others, (1994), cultures remained viable and continued to produce methane through five successive transfers, however they were unsuccessful at identifying any of the microorganisms.

The purpose of this research was to elucidate the diversity, composition, activity and function of the methane producing microbial community in coalbeds. The findings will have broader impacts than simply exploring the microbial ecology of a novel subsurface environment. Understanding the nature of the microbial ecology of coal beds will contribute knowledge toward management of enhanced microbial methane production and recovery, and possibly contribute to $CO_2$ sequestration efforts thereby impacting greenhouse gas mitigation strategies.

# Methods

## Sample collection

Two microbial samples were collected in Wyoming from the Tongue River Member, Big George coal seam. The sites are in the Powder River watershed in east central Johnson County, Wyoming. The coal samples were collected during under reaming, using forward air rotary, of an already cased CBM well. The samples were gathered from the diverter pipe on the drill rig with a sample screen. In less than one minute the coal was inserted into an anaerobic chamber with an oxygen-consuming package and sealed. The coal samples were held in cold storage until they arrived at the laboratory at the University of Montana. A water-quality sample was collected at a nearby CBM discharge point from wells completed in the same coal seam, but different from the well where the coal samples were collected. The water-sample was submitted to the Montana Bureau of Mines and Geology analytical laboratory for analysis.

The upper coal sample was collected while reaming from 1,525 feet to 1,530 feet below ground surface. The second coal sample was collected just after reaching the base of coal (1,596 feet) while cleaning the borehole. Because the well was cased, neither coal sample contained material from farther up the bore hole, and appeared clean and in good condition.

## Molecular Analysis

### Nucleic Acid extraction

**Aqueous phase:** To increase the biomass for molecular analysis, cells were collected by filtration onto three separate 142-mm Supor (Pall Corporation, Ann Arbor, MI) 0.2 um membrane filters. Each filter received an approximate equal volume of groundwater (approximately 13 liters). Filters were placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI) and frozen at -80° C. Prior to genomic DNA extraction, the frozen filter was crushed thoroughly within the collection bag. Processing of total community DNA from the filter was carried out by the direct lysis method of Holben (1997) with minor modifications. Briefly, 20 ml of autoclaved extraction buffer (200 mM sodium phosphate buffer (NaPO4), 100 mM ethylenediamine tetra-acetate (EDTA) and 1.5% sodium dodecyl sulfate (SDS), pH 8.0) was added to sterile Oak Ridge tubes containing sterile glass beads (5 g of 0.2 mm and 5 g of 1 mm diameter) (Sigma Chemical Co., St. Louis, MO.). To this tube, one macerated filter was added, placed in a 70° C water bath for 30 minutes with frequent vortexing (5 minute intervals). Tubes were then placed on a reciprocal platform shaker and shaken on high (approximately 100 oscillations/minute) for 30 minutes at room temperature. Filter, particulate and cell debris were removed by centrifugation (Sovall RC 5B Plus with SS34 rotor) at 10,000 RPM (7,796 x g) for 10 minutes at 10° C. Supernatant was transferred to clean Oak Ridge tubes and incubated on ice for 30 minutes to precipitate the SDS, then centrifuged as above to pellet SDS. Liquid was transferred to new 50 ml tubes with addition of 10% volume 3 M sodium acetate (pH

5.2) and 2.5 volumes 100% cold ethanol. After overnight incubation at -20° C, nucleic acids were collected by centrifugation, as described above. Nucleic acid pellets were resuspended in approximately 1 ml of sterile deionized water and precipitated by addition of 2.5 volumes 100% cold ethanol and placed at -20° overnight. After centrifugation (as above) the resulting nucleic acid pellet was air dried and suspended in approximately 500 ul TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

**Solid phase:** Anaerobic coal samples were subjected to direct nucleic acid extraction, as well as used as inocula in both groundwater and growth media. Direct nucleic acid extractions were performed using Power Soil DNA Extraction Kits (Mo Bio, Solano Beach, CA) as per manufacture's suggestion. In addition to standard extractions, coal samples were subjected to further DNA purification which included the addition of chaotropic salts (6M guanidine HCl) with ethanol washes in combination with silicon binding matrices. This method has proven to be beneficial when attempting to amplify various environmental samples.

**PCR amplification**

Resulting DNA from both solid and aqueous phases was subsequently subjected to DNA amplification by the Polymerase Chain Reaction (PCR) using both generally conserved primers and methanogen specific primer sets 16S rDNA primers (536fc and 907r) as well as the methanogen-specific primer pair 23fc and 440r). Both of these primer sets provided sufficient amplification to generate adequate PCR product for Denaturing Gradient Gel Electrophoresis (DGGE) analysis. More recently, the genomic DNA was amplified with a additional primer sets (ME1 and ME2), which are specific for the *mcr* (methyl coenzyme M reductase) gene (alpha subunit). The expected product is approximately 750 kb. The result of this amplification gave correct size products, which were gel purified and are to be used to align with groups of other amplicons derived from Powder River Basin coal samples and associated aquifers. From these alignments "coal specific" methanogen primers will be constructed, which will be used to amplify any samples that are coal related.

**Denaturing Gradient Gel Electrophoresis (DGGE)**

As mentioned above, the first two sets of primers (ME1 and ME2) generated amplicons from PCR amplification that were subjected to further analysis by DGGE. This method of analysis separates double stranded DNA amplicons run in an acrylamide gel matrix based on sequence differences. The gel matrix also contains a linear gradient of urea and formamide which act in concert to induce denaturing of the DNA strand. In figure 2, (below) each individual band theoretically represents an individual organism.

**Sequence analysis**

Sequence analysis is the process of identifying an organism based on it genomic nucleotide content. Bands of PCR amplified DNA were selected from the DGGE analysis (figure 2), excised and used directly for cloning and DNA sequence analysis. PCR

products obtained using the ME1 and ME2 primer sets were used to generate direct clone libraries. All PCR products, whether cut from gels or derived directly from amplification, were subject to a blunt-end cloning procedure, in which the pT7Blue-3 plasmid vector was used with the Perfectly Blunt Cloning Kit (Novagen, Madison, WI), as per manufacture's suggestion. Putative plasmid clones were identified based on blue-white screening. Plasmid DNA was subsequently purified using Qiagen mini-prep kits (Qiagen, Valencia, CA) according to the manufacturer's specifications. Insert size of individual clones was confirmed by restriction fragment analysis using *Eco*RI. All confirmed clones were subjected to unidirectional DNA sequence analysis and sequence comparison to determine the best match to known sequences using the either the Ribosomal Database Project II website at http://www.cme.msu.edu/RDP/html/index.html. or a Blast search on the NCBI website.

### Culturing methods

Coal samples were also used as inocula. Reduced ground water and growth media were allowed to incubate in the dark at room temperature. The growth medium for culturing core samples and for growing anaerobic consortia consisted of a modified mineral salts solution (Fedorak and Hrudey, 1984). Cultures were incubated in a headspace gas of 20% $CO_2$ and 80% $N_2$. Bottles were sealed with butyl rubber stoppers and crimped down with aluminum seals and received 0.35 g $NaHCO_3$/100 ml. All anaerobic work was completed in an oxygen free atmosphere to ensure anaerobic conditions prevailed.

## Results

### Coal-aquifer water quality

Analytical results of the coalbed water-quality sample indicated the total dissolved solids concentration was 2,056 mg/L, specific conductivity was 3,275 umhos/cm$^2$, and the pH was 7.63. The water temperature was 21.4 C. The sodium concentration was 779 mg/L, bicarbonate concentration was 2,216.8 mg/L, the sulfate concentration was not detectable (less than 2.5 mg/L), iron concentration was 0.043 mg/L and nitrate (as N) was 0.146 mg/L. The sodium adsorption ratio was 25.5. The water quality is typical of CBM production water in the PRB.

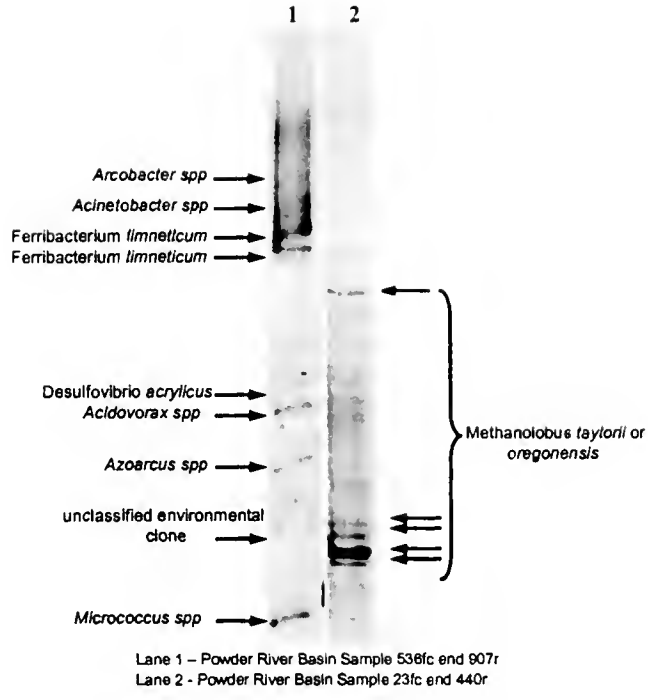


Figure 2. DGGE analysis of the microbial community of PRB coal associated aquifer water.

## Molecular-based analysis

The results indicate relatively low diversity of the total microbial community (see lane 1) compared to that of a typical subsurface or soil environment. Sequencing of several prevalent bands (indicated by arrows) indicated that all presumptive identities, based on known sequences in the Ribosomal Database (RDP II), had relevant metabolic capabilities consistent for their presumed role in coal formations that generate methane. The diversity of the methanogen community (as indicated by the number of bands in lane 2) appeared quite high, but the five bands sequenced all had similar phylogenetic affiliations. Each sequence was closely associated with the genus *Methanolobus* within the order *Methanosarcinales*. In addition, all five were most closely related to the species *M. taylorii* or *M. oregonensis* (averaging 93% homology). Interestingly and unexpectedly, this group is typically linked with marine environments, which may indicate that they thrive in a high sodium environment. However, the geological formation and shallow depth where this sample was taken have not been associated with ancient marine origins. This evidence supports the concept that this environment may sustain novel members of the methanogen group.

More recently, an additional survey of coal methanogens (using ME1 and ME2) produced seven sequences all related to members of the *Methanosarcinales* or *Methanobacteriales* orders. Six appear to be unique and their closest matches are to environmental clones from various origins (Table 1). This information will aid in development of primers specific for amplification of the methyl coenzyme M reductase gene from methanogens found in coal environments.

Many of the important members of the consortia may be underrepresented in terms of numbers, but may be dominant in terms of activity. If this is the case, it suggests that there are a number of minority microbial populations present in coalbeds, and that to more fully understand the community ecology an extensive and intensive investigation must be undertaken.

Table 1. Closest matched organism produced from the total microbial community extract and amplified using generally conserved 16S rDNA primers.

| Clone | Group Affiliation | Best Match Species | SAB Score | Functional in Environment |
|---|---|---|---|---|
| Meth 1 | Proteo -Epsil | Arcobacter | 0.075 | oxidize sulfur in aqueous environments |
| Meth 2 | Proteo-Gam | Acinetobacter | 0.92 | Environmental GW clone |
| Meth 3 | Proteo-Beta | Ferribacterium limneticum | 0.88 | Iron reducer |
| Meth 4 | Proteo-Beta | Ferribacterium limneticum | 0.91 | Iron reducer |
| Meth 5 | Proteo-Delta | Desulfovibrio acrylicus | 0.63 | SRB anoxic enviroments |
| Meth 6 | Proteo-Beta | Acidovorax | 0.89 | Denitrifying Iron - oxidizing bacteria |
| Meth 7 | Proteo-Beta | Azoarcus | 0.76 | N2-fixing plant- and fungus-associated |
| Meth 8 | Proteo-Epsil | unclassified clone | 0.78 | Environmental clone from activated sludge |
| Meth 9 | Proteo-Epsil | Arcobacter | 0.77 | isolated from a coculture capable of sulfate reduction |
| Meth 10 | Gram+ High GC | Micrococcus | 0.85 | Isolated from deep subsurface environment |

## Culture based analysis

In addition to the molecular data, coal samples were incubated in an anaerobic growth media used to specifically culture methanogens. To date, this has proven to be unproductive, with no apparent evidence of growth, based on turbidity and direct observations. This is not a complete surprise as methanogen growth is typically very slow and may indeed take a period of time well beyond the time frame of this study. However these culture attempts will continue and a molecular analysis of these samples will be undertaken. Data from this experiment may aid in future studies.

## Direct counts

Initial direct cell counts were completed on the coal samples by acridine orange (AOD) staining and were inconclusive. This was likely due to the fact that the coal contained an abundance of material that interfered with the staining of cells. Therefore differentiating cells from background material was very difficult. To improve on this, specific probes may be used in a fluorescent *in situ* hybridization (FISH) technique.

# Conclusions and recommendations

This initial investigation proved to be an excellent starting point for continuing efforts toward unraveling the complexity of the microbial community responsible for biogenic methane production. In this study we found a lower-than-expected total microbial community diversity. All of these identified community members were presumptively capable of metabolic processes leading to the formation of methane. In regards to methanogens, the organisms grouped fairly coherently into two orders (*Methanosarcinales* and *Methanobacteriales*). Because so little is known about methanogens in coal beds we can not yet identify their specific role in methane production, other then to say that, indeed methanogens are prevalent in our coal and associated samples.

To fully underpin the microbial community in this environment a more comprehensive study must be undertaken which would include the following:

- Continue with sequencing efforts on the total microbial community within coal samples (not limited to methanogens).
- Develop conceptual models of the microbial community present based on molecular analysis.
- Design primers appropriate for real-time PCR for measuring abundance of particular functional groups associated with coal.
- Conduct culturing experiments and isolate pure cultures to confirm the presence of novel organisms (methanogens as well as other major groups)
- Design amendment/perturbation experiments for laboratory (later for *in situ*?)
- Develop activity studies aimed at determining active microbial populations responsible for methane production (future stable isotope experiment)

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